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BIOCONTROL OF PYTHIUM DAMPING-OFF ON PEPPER (*CAPSICUM ANNUUM*) WITH SELECTED FUNGAL AND RHIZOBACTERIAL AGENTS

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ABSTRACT

Pythium ultimum is a common soilborne pathogen causing serious losses of pepper seedlings in nurseries and few weeks post-planting. Two pepper associated-*P. ultimum* isolates (P1 and P2) were shown pathogenic to pepper cv. Altar causing post-emergence damping-off with P2 isolate being the most aggressive. Fungal and bacterial antagonists have been evaluated *in vitro* and *in vivo* for their ability to suppress *P. ultimum*. In dual culture assay, *Trichoderma harzianum*, *T. viride* and *Gliocladium virens* inhibited pathogen radial growth by 18.54, 17.52 and 15.24%, respectively, relative to control, while none of the tested bacteria was shown able to significantly inhibit pathogen growth. However, drastic changes in pathogen hyphae expressed as strong lysis, the formation of mycelial cords and mycoparasitism have been observed. Pepper seeds treated with fungal antagonists' conidial suspensions showed 60, 50 and 60% less pre-emergence damping-off infections, respectively, compared to the positive control. When tested as root dipping, only *G. virens* resulted in 40% reduced post-emergence damping-off. An improved seedlings fresh weight, by 79.31 and 76%, was respectively induced by *G. virens*-, and *T. viride*-based treatments while an increment of 27.58, 25.33 and 22.22 % was recorded following treatments with *G. virens*, *T. viride* and *T. harzianum*, relative to the positive control. The majority of tested bacterial isolates, applied as a seed treatment, had significantly improved the emergence percentage of inoculated seedlings as compared to control with *Burkholderia glathei* isolate 35 being the most efficient. When applied as root dipping, reduction of post-emergence damping-off ranged between 40 and 100% with *Pseudomonas aureofaciens* isolate 314 being the most effective agent. Seedlings treated with *P. aureofaciens* (314) and *Bacillus pumilus* (420) showed 35.38 and 28.51% higher heights, respectively. Plant weight was enhanced by 73.06, 61.18, 77.39, 61.8 and 67.93% over control following treatments with *P. aureofaciens* isolates 314 and 31, *Bacillus pumilus* 420, *P. fluorescens* and *P. putida* 227.

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INTRODUCTION

In Tunisia, pepper (*Capsicum annuum* L.) is an economically important crop coming right after tomato and potato in terms of cropped vegetable areas, of about 20 000 ha (Anonymous, 2019), and with an average production of about 346 000 tons during the last five

years (Anonymous, 2019). It is widely grown in almost all Tunisian regions both on open fields, for season and late season crops, and under plastic greenhouses for the off-season product (Zhani *et al.*, 2012). These crops ensure a continuous supply of pepper market and Tunisia ranking as one of the major of pepper producers and exporters in Africa (STAT, 2013).

However, soilborne fungal and fungal-like diseases are major yield limiting factors in pepper production in Tunisia and worldwide. Among them, damping-off is a serious disease complex which involves germination failure, prevention of seedling emergence after germination, or the rotting and collapse of seedlings at the soil level (Lamichhane *et al.*, 2017). *Pythium* spp. *Fusarium* spp., *Rhizoctonia* spp., and *Phytophthora* spp. are the most frequently important pathogens associated with vegetable crops such as pepper.

Two types of the damping-off disease occur in plants susceptible to *Pythium*, pre-emergence and post-emergence which are economically important worldwide (Whipps and Lumsden, 1991). *Pyhtium* species responsible for pre-emergence damping-off can attack seeds or seedlings below the soil line before emergence, while post-emergence damping-off symptoms occur when seedlings decay, wilt, and die just after emergence (Lamichhane *et al.*, 2017).

Pythium species can cause more than 60% seedling mortality both in nursery and in main field (Manoranjitham *et al.*, 2000). In addition to damping-off disease, *Pythium* spp. can cause wilt in older seedling and mature plants. Yield loss due to these pathogens in different crops has been estimated approximately of multibillion dollar worldwide (van West *et al.*, 2003). Among *Pythium* species, *P. ultimum* is one of the most pathogenic and problematic species that cause seed and seedling losses in nurseries in the first weeks after sowing (Whipps and Lumsden, 1991).

Given the complex nature of Pythium damping-off disease and the numerous factors involved in its occurrence, its management is still very difficult. In fact, due to the soilborne nature of these microorganisms, their wide host range, the prolonged survival of their propagules in the soil, the lack of resistant cultivars and the ability of *Pythium* spp. to develop resistance against recommended pesticides, strategies to control Pythium damping-off diseases are limited (Li *et al.*, 1995).

Continuous efforts have been emphasized on developing effective biocontrol agents for the management of these diseases, which are considered as economically viable alternatives, ecologically sustainable and safe crop protection solutions (Khare and Upadhyay, 2009; Muthukumar *et al.*, 2011).

Considerable research has been done to investigate the potential that offers various microbial agents including filamentous fungi, yeast, bacteria and actinomycetes, for the biocontrol of Pythium damping-off. In the last three decades, several antagonists were used against *Pythium* spp., for reducing their growth and preventing their establishment in the rhizosphere (Howell, 2003).

Various fungal agents belonging to *Trichoderma* and *Gliocladium* genera i.e. *Gliocladium virens*, *Trichoderma harzianum* and *T. hamatum* are among the most commonly studied biocontrol agents which effectiveness has been demonstrated against not only *P. ultimum* (Naseby *et al*., 2000) but also towards several damping-off causative pathogens such as *R. solani* (Lewis and Papavizas, 1987; Mannai *et al.*, 2018). The successful application of these species for the management of damping-off in chili pepper and tomato has been previously reported (Jayaraj *et al.*, 2006; Muthukumar *et al.*, 2011). The combination of the biocontrol agents, *Pythium nunn* and *T. harzianum* isolate T-95 reduced Pythium damping-off disease in cucumber grown under greenhouse conditions (Paulitz *et al.*, 1990). *G. virens* was among the most consistent and effective agents in controlling damping-off of zinnia, cotton, and cabbage seedlings incited by *P. ultimum* (Lumsden, 1989).

Several bacterial species belonging to *Pseudomonas, Bacillus, Burkholderia* and *Streptomyces* genera have been also used to manage *Pythium*-induced diseases on pepper and many other vegetable crops (Naseby, Pascual*, et al.*, 2001; El-Mohamedy, 2012; Khabbaz and Abbasi, 2014). Numerous studies have demonstrated the potential of different *P. fluorescens* strains as biocontrol agents of *P. ultimum* (Ramamoorthy *et al.*, 2002; Carisse *et al.*, 2003). For instance, *P. fluorescens* isolate Pf1 was effective in reducing the damping-off incidence in tomato and hot pepper crops grown under greenhouse and field conditions (Ramamoorthy *et al.*, 2002). Hultberg *et al.* (2000) studies recorded the biocontrol potential of specific strains of *P. fluorescens* against damping-off of tomato seedlings. Gravel *et al.* (2005) showed the efficiency of *P. fluorescens* to suppress tomato damping-off caused by either *P. ultimum* or *P. aphanidermatum* in Rockwool. Two *B. subtilis* strains were shown effective against *P. ultimum* and exhibited

effective control of damping-off on cauliflower (Abdelzaher, 2003).

Presently, in Tunisia, none of the pepper cultivars used in greenhouse or open field crops showed satisfying levels of resistance to *Pythium* spp. infections and no biocontrol studies have been done for this pathogen. Therefore, the objectives of the present study were: (i) to evaluate the virulence of two *P. ultimum* isolates involved in pepper damping-off (ii) to select the most effective fungal and bacterial isolates in suppressing *P. ultimum* mycelial growth and pepper damping-off disease and in improving plant growth.

MATERIALS AND METHODS

Plant Material

Pepper cv. Atlar seeds and seedlings were used in this study. This cultivar was widely used in Tunisia. After surface disinfection with 5% sodium hypochlorite solution for 5 min, three rinsings with sterile distilled water (SDW) and air drying, pepper seeds were sown in 77 cell-plates containing sterilized peat and kept under greenhouse conditions for 30 days. Seedlings were watered as needed.

Pathogen Culture and Inoculum Preparation

Two characterized *P. ultimum* isolates recovered from pepper seedlings exhibiting typical damping-off symptoms were used in the present study (Table 1). Root samples (1-2 cm in length) were surface-disinfested in sodium hypochlorite solution (3%) for 2 min, cut in small pieces (0.5 cm in length), were rinsed three times with SDW, dried on sterile filter paper and plated onto Potato Dextrose Agar (PDA) medium amended with streptomycin sulphate (100 mg/L) (w/v). Fungal cultures were incubated for 7 days at 25°C. *Pythium* growing colonies were cleaned up by successive sub-culturing. Pathogenicity of *Pythium* isolates collected was confirmed on pepper cv. Atlar seedlings fulfilling Koch's postulates. Before use, *Pythium* isolates were cultured in the dark for 6 days at 25°C on PDA medium.

P. ultimum inoculum was prepared by collecting mycelia and sporangia from five 6-day-old cultures grown on PDA and mixing them in 0.5 L of SDW. The resulting mycelial fragments and sporangia served for substrate inoculation.

Fungal and Bacterial Biocontrol Agents

Trichoderma harzianum (TH), *T. viride* (TV) and *Gliocladium virens* (GV) were the three selected biocontrol agents to be used in the current study (Table 2). These fungal agents, originally recovered from Tunisian soils, were selected based on their antifungal potential previously demonstrated against various soilborne plant pathogens such as *Verticillium* spp., *Fusarium* spp., and *R. solani* (Ayed *et al.*, 2006; Jabnoun-Khiareddine *et al.*, 2009; Soumaya *et al.*, 2013).

Table 1. *Pythium ultimum* isolates used in this study.

Isolates	Original host	Cultivar	Isolation site
P1	*Capsicum annuum*	Beldi	Chott-Mariem
P2	*C. annuum*	Baklouti	Sahline

Table 2. Rhizobacterial isolates used in this study.

Isolate codes	Bacterial species	Origin
Pf	*Pseudomonas fluorescens*	Tunisia (a reference bacterium)
263	*Bacillus subtilis*	Tunisia
227	*P. putida*	Tunisia
31	*P. aureofaciens*	Tunisia
420	*B. pumilus*	Missouri
35	*Burkholderia glathei*	Missouri
314	*P. aureofaciens*	Missouri
69	*P. huttiensis*	Missouri

To prepare biological treatments, mycelium from 7-day-old cultures grown on PDA medium was scraped off, homogenized with SDW, and then filtered through two layers of muslin. The resulting conidial suspension was adjusted to 10^7 spores/mL. Eight rhizobacterial isolates belonging to three genera, namely *Pseudomonas*, *Bacillus* and *Burkholderia*, were tested in this study (Table 2). They were isolated and identified in previous work

(Nasraoui *et al.*, 2007). Bacterial stock cultures were maintained at -20°C in Nutrient Agar (NA) amended with 40% glycerol. Before use, bacterial isolates were grown on NA and incubated at 25°C for 48 h. Bacterial cell suspensions used for *in vitro* and *in vivo* bioassays were prepared by scraping bacterial colonies, previously grown in NA for 48 h, in SDW and adjusted to 10^6 cells/mL.

Pathogenicity Tests

P. ultimum isolates were challenged to pepper cv. Atlar seedlings to test their ability to cause damping-off disease. Thirty days-old pepper seedlings were transplanted in cell trays filled with sterilized peat mixed with *P. ultimum* inoculum at the rate of 1:3 (v/v). Seedlings transplanted in uninoculated peat were used as an untreated control. The experiment was conducted in a completely randomized design. Five pepper seedlings were used for each pathogen isolate. Trays were then incubated at 26/18°C (day-night temperatures).

At five days post-transplanting, damping-off incidence, estimated as the percentage of wilted pepper seedlings, was noted for each *Pythium* isolate. Plant height (cm) and plant fresh weight (g) were also determined.

***In vitro* Antagonism Assay**

The ability of fungal and bacterial agents to inhibit *P. ultimum in vitro* growth was assessed using the dual culture technique. Agar plugs (6 mm diameter) cut from 7-day-old pathogen cultures and fungal colonies were placed opposite to each other at the peripheries of 9-cm Petri plate containing PDA. Six plates were used per each individual treatment and the experiment was repeated twice.

For the screening of bacterial agents, dual confrontation was performed by depositing two agar plugs (6 mm in diameter) carrying the pathogen equidistantly with respect to the tested bacterial agent seeded in the form of a line carried along the axis of the plate using a sterile rod soaked with the bacterial suspension. Control plates were challenged with pathogen plugs and the bacterial suspension was replaced by SDW. Three plates were used per individual treatment and the experiment was repeated twice.

All cultures were incubated at 25 °C for 2 days. The diameter of the pathogen colony was measured, together with macroscopic and microscopic observations made to characterize the *in vitro* pathogen-antagonist interactions more focused on hyphal alterations. Percentage growth inhibition (%) of the pathogen was calculated according to the following formula: Growth inhibition % = [(dc-dt)/ dc] ×100, Where dc = Colony diameter in control plates; dt = Colony diameter in treated plates.

***In vivo* Biocontrol Trials**

Two biocontrol trials were carried out to evaluate the ability of the tested fungal and bacterial agents in reducing pre- and post-emergence damping-off, incited by *P. ultimum*.

Evaluation of Pre-Emergence Damping-Off Suppression Ability

Ten pepper cv. Atlar seeds were soaked for 10 min in each antagonist suspension prepared as previously described. Treated seeds were sown in cell trays filled with sterilized peat mixed with the inoculum of the virulent *P. ultimum* isolate (P2) at a rate of 1:3 (v/v). Trays were then kept at room temperature (25-30 °C).

Pre-emergence damping-off percentage was noted 15 days post-treatment, based on the number of non-emerged seeds in relation to the number of total sown seeds as follow:

Pre-emergence damping %= germination in untreated control–germination in treatment/ germination in untreated control.

Evaluation of Post-Emergence Damping-Off Suppression Ability

Pepper cv. Altar seedlings (30-day-old) were treated by root soaking in the antagonist suspension for 30 min. Then, treated seedlings were transplanted in cell trays filled with peat previously infected with *P. ultimum* isolate P2 inoculum at the rate of 1:3 (v/v). Control seedlings were root soaked in SDW and transplanted in pathogen-inoculated and pathogen-free substrates (for positive and negative controls, respectively). All treated seedlings were incubated under growth chamber conditions (at 27-30/15-18°C day-night temperatures). Five repetitions were used per each individual treatment.

Three parameters were recorded five days post-transplanting: (i) plant height, (ii) plant fresh weight and (iii) post-emergence damping-off percentage. Post-emergence damping-off (%) was calculated as follow: Post-emergence damping-off (%) = number of infected seedlings in untreated control–number of infected

seedlings in treatment/ number of infected seedlings in the untreated control.

Statistical Analysis

Results from the current study were subjected to one-way analysis of variance and means separations were carried out using the Student-Newman-Keuls (SNK) test at $P \leq 0.05$. ANOVA was performed using the Statistical Package Social Sciences (SPSS) software version 20.0. Experiments were conducted according to a completely randomized design for *in vitro* (6 replicates), pre-emergence damping-off (10 replicates) and post-emergence damping-off trials (5 seedlings per individual treatment).

RESULTS

Pathogenicity of *Pythium ultimum* Isolates

At five days post-transplanting in *Pythium*-inoculated peat, all pepper cv. Altar seedlings exhibited typical damping-off symptoms with varying incidence depending on the isolate used for inoculation whereas the uninoculated control (NIC) seedlings remained symptomless. P2 isolate induced complete death of all pepper seedlings (Figure 1) compared to only 40% noted on P1-inoculated seedlings.

As given in Table 3, only P2 isolate significantly ($P \leq 0.05$) reduced plant weight and height by 66.66 and 22.72%, respectively, compared to pathogen-free control.

Figure 1. Comparison between a pepper cv. Altar seedling inoculated with *Pythium ultimum* P2 isolate (at right) and an uninoculated control (NIC) one (at left).

Table 3. Effects of *Pythium ultimum* isolates on damping-off incidence and some growth parameters of pepper cv. Altar noted five days post-inoculation.

Treatments	Damping-off incidence (%)	Plant weight (g)	Plant height (cm)
NIC	0.00 ±0.00 a	0.60±0.04 a	4.40±0.07 a
P1	40.00±0.00 b	0.52±0.12 a	4.16±0.19 a
P2	100.00±0.00 c	0.20±0.03 b	3.40±0.23 b

Within each column, values followed by the same letter are not significantly different according to SNK test at $P \leq 0.05$.

***Pythium ultimum* Biocontrol Using Fungal and Bacterial Bio-Agents**

***In vitro* Biocontrol Activity Displayed by Fungal Agents**

P. ultimum mycelial growth, noted after six days of incubation at 25 °C, varied significantly ($P \leq 0.05$) depending on fungal treatments tested. *T. harzianum*, *T. viride*, and *G. virens* reduced pathogen radial growth by 18.54, 17.52, and 15.24%, respectively, relative to the untreated control (Figure 2). Moreover, these antagonists grew and sporulated abundantly, invaded *P. ultimum* colonies and reach their opposite edge after six days of dual culture at 25 °C (Figure 3).

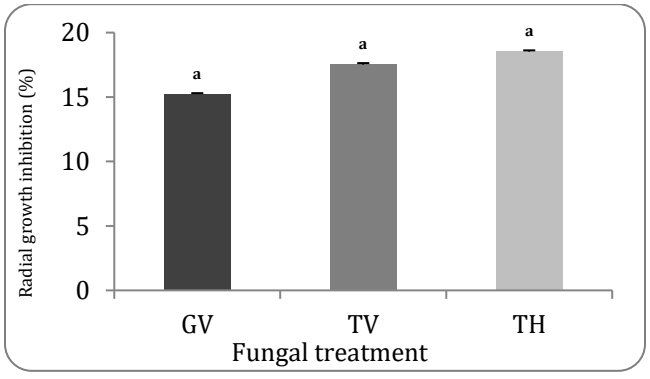

Figure 2. Radial growth inhibition of *Pythium ultimum* noted after two days of dual culture with fungal antagonists as compared to control.

Microscopic observations made at the contact zone between *P. ultimum* and the biocontrol agents colonies revealed the formation of mycelium cords (Figure 4a) and the coiling of *T. harzianum*, *T. viride* and *G. virens* mycelia around pathogen mycelium (Figure 4b).

In vitro Biocontrol Activity Displayed by Rhizobacterial Agents

P. ultimum radial growth, noted after two days of incubation at 25 °C, did not vary significantly depending on tested bacterial treatments as compared to control. However, microscopic observations made at the contact zone between the tested rhizobacteria and *P. ultimum* showed hyphal lysis (Figure 5a) and formation of mycelial cords as a stress response to these biological treatments (Figure 5b).

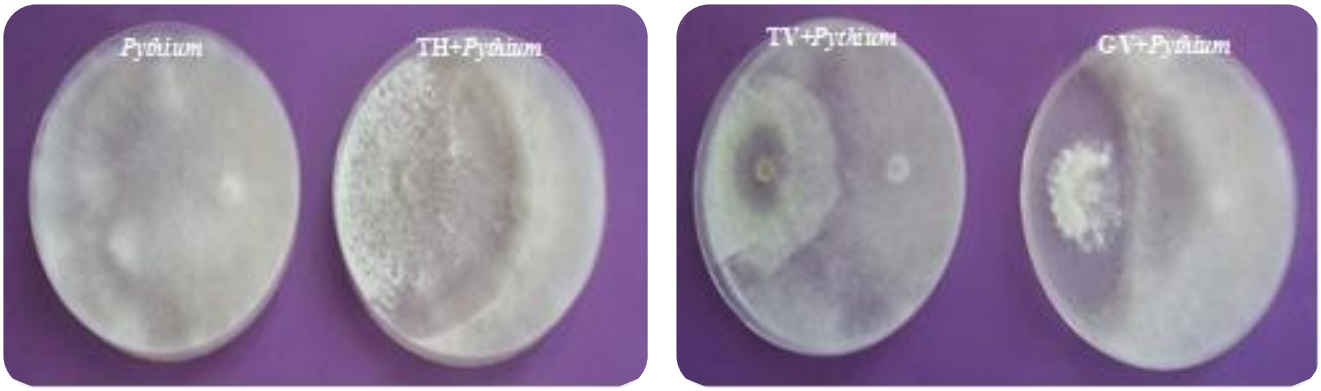

Figure 3. Antagonistic potential of *Trichoderma harzianum* (TH), *Gliocladium virens* (GV) and *T. viride* (TV) dual cultured with *Pythium ultimum*, noted two days after incubation at 25 °C, as compared to pathogen-inoculated and untreated control.

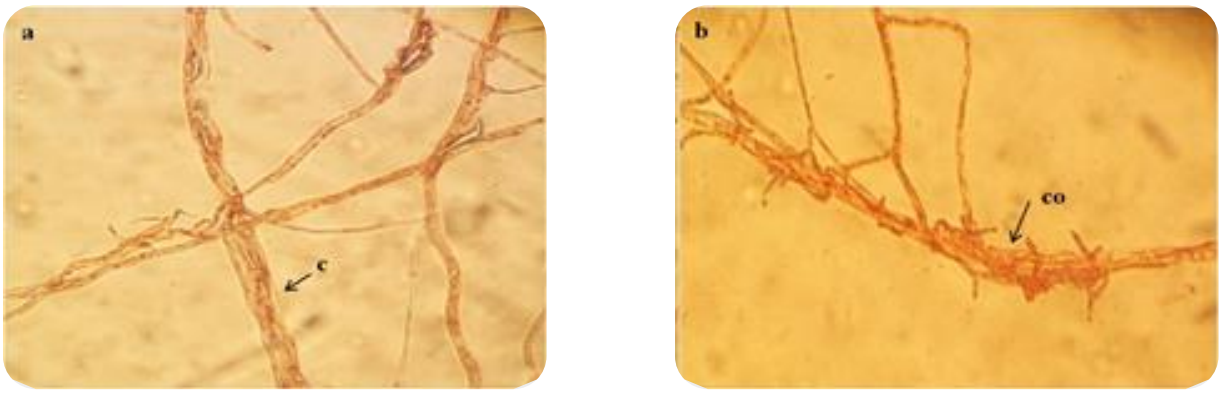

Figure 4. Hyphal interactions at the confrontation zone between *Pythium ultimum* and *Trichoderma* spp.: (a) Mycelial cords of *P. ultimum* (c) treated with *T. harzianum*; (b) Coiling (co) of *T. viride* hyphae around pathogen mycelium (×400).

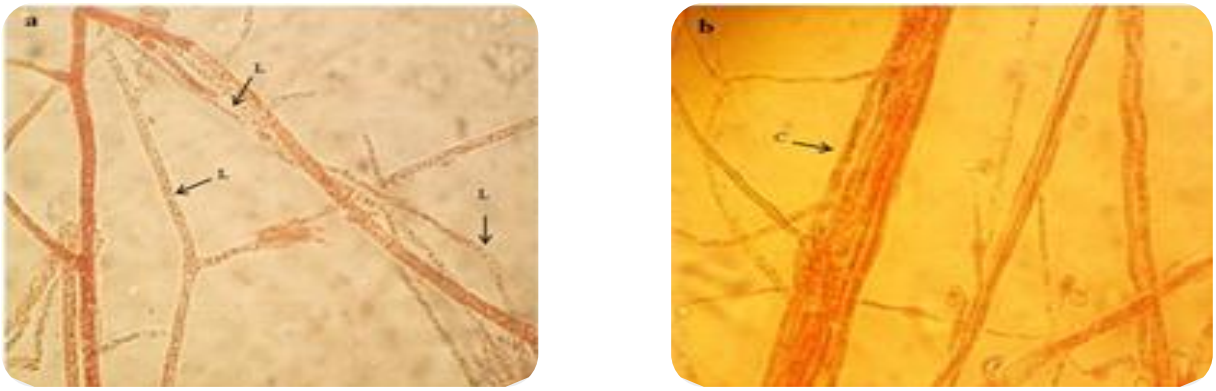

Figure 5. *Pythium ultimum* mycelium lysis (a) and formation of mycelial cords (b) noted at the confrontation zone with of *Burkholderia glathei* 35 (a) and *Pseudomonas aureofaciens* 31 (b). L: Mycelial lysis; C: Mycelial cords (×400).

Biocontrol of Pepper *Pythium* Damping-Off Using Fungal Antagonists

Suppression of Pre-Emergence Damping-Off

The treatment of pepper cv. Altar seeds with the tested fungal antagonists improved the percentage of seedling emergence noted after 15 days of incubation, compared to the pathogen-inoculated and untreated control. *P. ultimum* pre-emergence damping-off was suppressed by 75, 62.50 and 75%, with *G. virens*, *T. viride* and *T. harzianum* based treatments, respectively, as compared to pathogen-inoculated and untreated control (Figure 6).

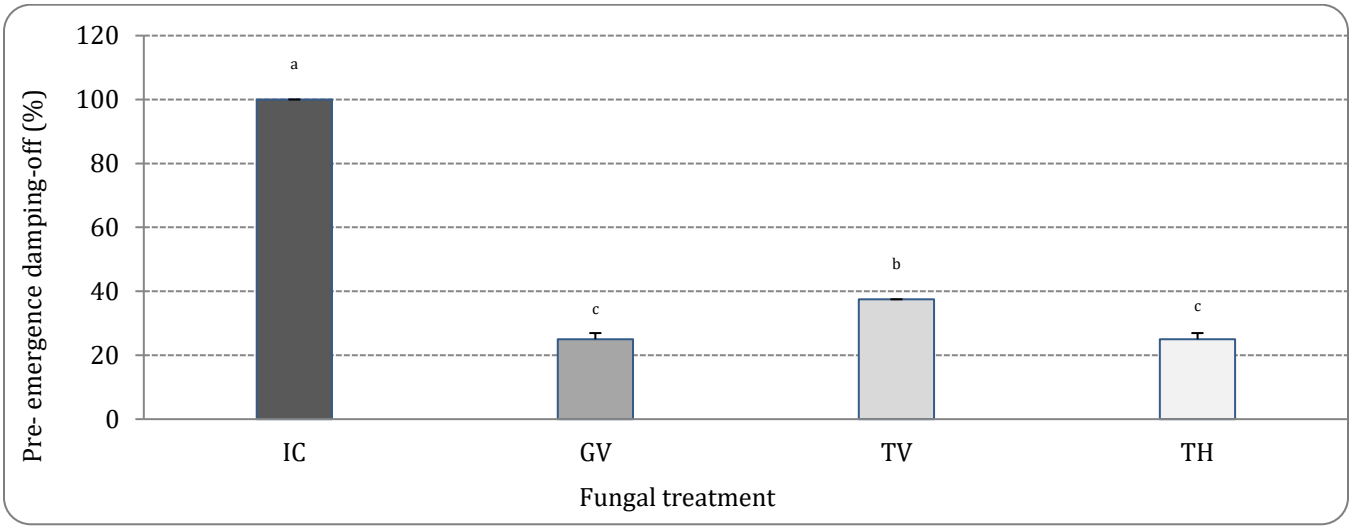

Figure 6. Pre-emergence damping-off (%) of pepper cv. Atlar seedlings inoculated with *Pythium ultimum* and treated with fungal antagonists, noted 15 days post-inoculation, as compared to pathogen-inoculated and untreated control. Bars sharing the same letter are not significantly different according to SNK test ($P \leq 0.05$).
IC: Inoculated with *Pythium ultimum* and untreated control; GV: Inoculated and treated with *Gliocladium virens*; TV: Inoculated and treated with *Trichoderma viride*; TH: Inoculated and treated with *T. harzianum*.

Suppression of Post-Emergence Damping-Off

Pepper cv. Altar seedlings treatment with *G. virens* resulted in 40% reduced infection compared to the pathogen-inoculated and untreated control, while the other fungal treatments did not significantly suppress post-emergence damping-off, 5 days after inoculation (Table 5).

Growth parameters noted on pepper cv. Altar seedlings 5 days post-transplanting, differed significantly ($P \leq 0.05$) upon tested treatments (Table 5). *G. virens*-, and *T. viride*-based treatments significantly increased plant fresh weight of *P. ultimum*-inoculated plants, by 79.31 and 76%, respectively, compared to pathogen-inoculated and untreated control (Figure 7). In addition, height noted of inoculated and treated seedlings were significantly compared to that of the uninoculated and untreated ones (Table 5).

Figure 7. Comparison between pepper cv. Altar seedlings inoculated with *Pythium ultimum* and treated with *Gliocladium virens* (GV), pathogen-inoculated (IC), and uninoculated and untreated (NIC) controls, noted 5 days post-treatment.

Table 5. Damping-off incidence and growth parameters noted 5 days post-treatment on pepper cv. Altar seedlings inoculated with *Pythium ultimum* and treated with different fungal antagonists as compared to controls.

Treatments	Damping-off incidence (%)	Plant weight (g)	Plant height (cm)
NIC	0.00±0.00 a[×]	0.61± 0.02 a	4.46± 0.18 a
IC	50.00±0.00 b	0.12± 0.02 b	3.36± 0.19 b
GV	10.00±0.00 a	0.58± 0.02 a	4.64± 0.12 a
TV	40.00±0.00 b	0.50± 0.03 a	4.50± 0.12 a
TH	50.00±0.00 b	0.21± 0.05 b	4.32± 0.27 a

[×] Within each column, values followed by the same letter are not significantly different according to SNK test ($P \leq 0.05$).
NIC: Uninoculated control; IC: Inoculated with *Pythium ultimum* and untreated control; GV: Inoculated and treated with *Gliocladium virens*; TV: Inoculated and treated with *Trichoderma viride*; TH: Inoculated and treated with *T. harzianum.*

Plant height varied significantly ($P \leq 0.05$) depending on tested treatments. All tested biological treatments had significantly improved this parameter by 27.58% (for *G. virens*), 25.33% (for *T. viride*) and 22.22% (for *T. harzianum*) over pathogen-inoculated and untreated control. It is also to note that the height of *G. virens*-treated, and pathogen-inoculated seedlings was improved by 3.9% over the uninoculated and untreated control ones (Table 5).

Biocontrol of Pepper *Pythium* Damping-Off Using Rhizobacterial Agents

Suppression of Pre-Emergence Damping-Off

At 15 days post-inoculation, all tested rhizobacterial isolates, excepting *B. subtilis* 263, *P. fluorescens* and *P. putida* 227, had improved the emergence percentage of *P. ultimum*-inoculated seedlings as compared to pathogen-inoculated and untreated control. The recorded increment reached 50% following treatment with *Burkholderia glathei* 35 (Figure 8).

Suppression of Post-Emergence Damping-Off

All tested bacterial treatments reduced post-emergence damping-off on pepper cv. Altar seedlings already infected with *P. ultimum* as compared to pathogen-inoculated and untreated control. The recorded reduction ranged from 100% for seedlings treated with *P. aureofaciens* 314 to 40% for those treated with *Burkholderia glathei* 35 and *P. huttiensis* 69. Compared to the reference strain, *P. fluorescens* (Pf), *P. aureofaciens* 314 and *B. pumilus* 420 were found to be more effective in suppressing pepper *P. ultimum* damping-off (Table 6).

As shown in Table 6, plant height, noted 5 days post-treatment, varied significantly upon bacterial treatments tested. Plant height, noted on pepper seedlings treated with *P. aureofaciens* 314 and *B. pumilus* 420, was improved by 35.38 and 28.51%, respectively, relative to positive control whereas those treated with the remaining isolates showed similar heights as both controls (Table 6).

Table 6. Damping-off incidence and growth parameters noted on pepper cv. Altar seedlings inoculated by *Pythium ultimum* and treated with different rhizobacterial antagonists as compared to controls noted 5 days post-treatment.

Treatment	Damping-off incidence (%)	Plant weight (g)	Plant height (cm)
NIC	0.00± 0.00 a[×]	0.61± 0.02 a	4.46± 0.18 abc
IC	50.00± 0.00 d	0.12± 0.02 e	3.36± 0.19 c
314	0.00± 0.00 a	0.44± 0.04 bc	5.20± 0.21 a
35	30.00± 0.00 c	0.20±0.01de	3.54± 0.12 bc
31	20.00± 0.00 bc	0.30± 0.04 cd	4.06± 0.21 abc
420	10.00± 0.00 ab	0.52± 0.03 ab	4.70± 0.12 ab
69	30.00± 0.00 c	0.19± 0.03 de	4.28± 0.16 abc
263	20.00± 0.00 bc	0.08± 0.01 e	3.32± 0.10 c
Pf	20.00± 0.00 bc	0.29± 0.04 cd	4.28± 0.40 abc
227	0.00± 0.00 a	0.37± 0.04 cd	4.14± 0.21 abc

[×] Within each column, values followed by the same letter are not significantly different according to SNK test ($P \leq 0.05$).
NIC: Uninoculated control; IC: Inoculated with *Pythium ultimum* and untreated control; 227: Inoculated and treated with *Pseudomonas putida* 227; 420: Inoculated and treated with *Bacillus pumilus* 420; 69: Inoculated and treated with *P. huttiensis* 69; 31 and 314: Inoculated and treated with *P. aureofaciens* 31 and 314; 35: Inoculated and treated with *Burkholderia glathei* 35; 263: Inoculated and treated with *B. subtilis* 263; Pf: Inoculated and treated with *P. fluorescens.*

As for their effects on pepper plant weight, analysis of variance revealed the presence of a highly significant difference ($P \leq 0.01$) between tested bacterial treatments. Seedlings inoculated with *P. ultimum* and treated with *P. aureofaciens* 314, *P. aureofaciens* 31, *Bacillus pumilus* 420, *P. fluorescens* and *P. putida* 227 showed 73.06, 61.18, 77.39, 61.8 and 67.93% higher fresh weights, respectively, relative to pathogen-inoculated and untreated control (Table 6). As illustrated in Figure 9, *P. aureofaciens* 314 was able to enhance plant growth and to reduce the post-emergence damping-off relative to *P. ultimum*-inoculated and untreated control.

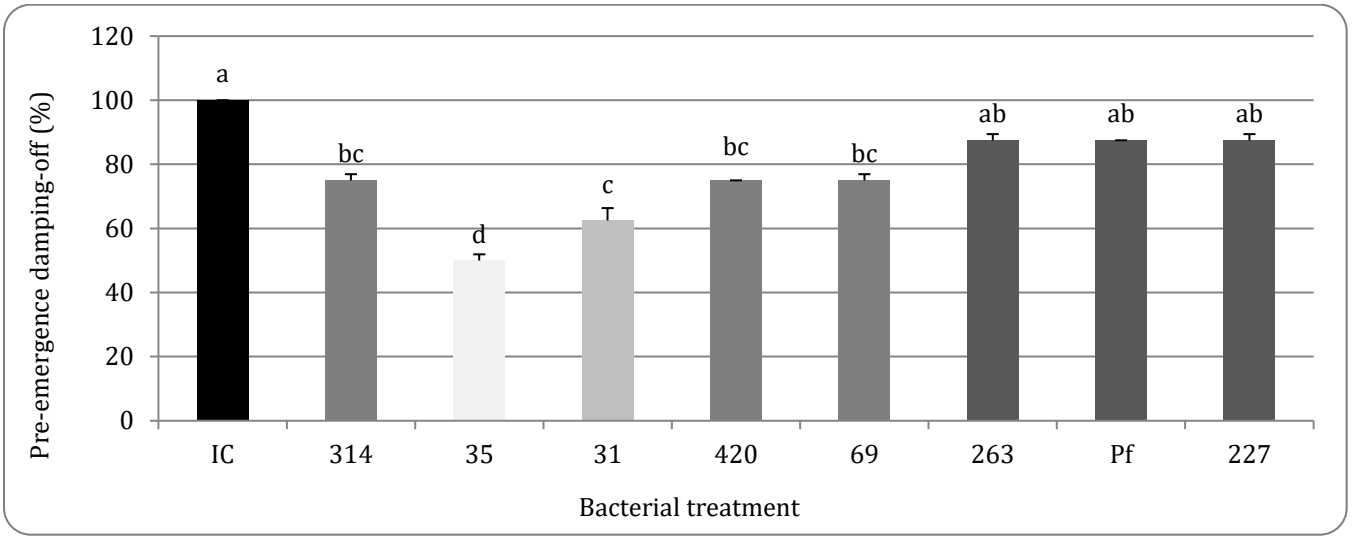

Figure 8. Pre-emergence damping-off of pepper cv. Atlar seedlings inoculated with *Pythium ultimum* and treated with different rhizobacterial isolates, noted 15 days post-inoculation, as compared to inoculated control.
Bars sharing the same letter are not significantly different according to SNK test ($P \leq 0.05$).
IC: Inoculated with *Pythium ultimum* and untreated control; 227: Inoculated and treated with *Pseudomonas putida* 227; 420: Inoculated and treated with *Bacillus pumilus* 420*;* 69: Inoculated and treated with *P. huttiensis* 69*;* 31 and 314: Inoculated and treated with *P. aureofaciens* 31 and 314; 35: Inoculated and treated with *Burkholderia glathei* 35; 263: Inoculated and treated with *B. subtilis* 263*;* Pf: Inoculated and treated with *P. fluorescens.*

DISCUSSION

In the current investigation, two *P. ultimum* isolates were shown pathogenic to pepper cv. Altar causing post-emergence damping-off. P2 isolate was found to be the most virulent and able to decrease plant weight and height by 66.44% and 22.72% respectively, as compared to the uninoculated control. These findings are also in agreement with previous studies reporting the pathogenicity of *P. ultimum* on pepper (Ramamoorthy *et al.*, 2002) as well as on different crops like bean (Rossman *et al.*, 2017), tomato (Rafin and Tirilly, 1995), pea (Naseby, Way*, et al.*, 2001), cabbage (Tojo *et al.*, 2005), Broccoli (El-Mohamedy, 2012), and sorghum (Idris *et al.*, 2008). The rapid germination of *Pythium* sporangia exposed to root exudates or volatiles from seeds (Osburn, 1989) and its direct infection, makes very difficult pathogen control (Whipps and Lumsden, 1991). In last three decades, the use of fungal and bacterial biocontrol agents has offered a potential and viable solution to control damping-off. Increasing attention has been paid to biological control through the use of antagonistic fungi belonging to *Trichoderma* and *Gliocladium* genera and different bacterial species affiliated to *Bacillus* and *Pseudomonas* genera (El-Mohamedy, 2012; Khabbaz and Abbasi, 2014; Gravel *et al.*, 2005).

Results from the present study showed that *Pythium ultimum* radial growth was inhibited, after two days of dual culture, with *T. harzianum, T. viride* and *G. virens*. Although this inhibition did not exceed 18%, the hyphal *in vitro* interactions between *P. ultimum* and tested fungal antagonists resulted in severe alterations of pathogen hyphae. The tested antagonists showed mycoparasitic abilities towards *P. ultimum* which was demonstrated at the confrontation zone. *Trichoderma* species have developed various mechanisms for attacking other fungi including mycoparasitism (Haran *et al.*, 1996), production of inhibitory compounds (Sivasithamparam and Ghisalberti, 1998), competition

for space and nutrients (Elad *et al.*, 1999), inactivation of the pathogen's enzymes. Harman *et al.* (1980) have also suggested that mycoparasitism is the main mechanism involved in Pythium damping-off control when seeds were coated with *Trichoderma hamatum*. Our results are also in concordance with those of Daami-Remadi (2001), Kerkeni *et al.* (2007), El-Katatny *et al.* (2006), and El-Mohamedy (2012), showing the efficacy of these antagonistic species against *P. ultimum* mycelial growth.

It is also interesting to note that treating pepper cv. Atlar seeds with *G. virens*, *T. viride* and *T. harzianum* had reduced the incidence of the pre-emergence damping-off disease incited by *P. ultimum* by 60, 50, and 60%, respectively, as compared to pathogen-inoculated and untreated control. When these treatments were applied as root soaking, only *G. virens* resulted in 40% reduced post-emergence damping-off. An improved pepper seedlings growth (height and fresh weight) was noted using these biological treatments as compared to the pathogen-inoculated and untreated control. These results are in agreement with numerous previous studies. *T viride* is effective in controlling Pythium cucumber damping-off (Kerkeni *et al.*, 2007). Besides, Harman (2000) reported that *T. viride* could colonize roots and promote plant growth. In addition, dipping roots of broccoli seedlings in water suspensions of *T. harzianum* and *T. viride* and mixing soil with the same suspensions of biocontrol agents during transplanting is efficient in reducing Pythium rot disease (El-Mohamedy, 2012). Moreover, *Trichoderma* species, added to the soil or applied as seed treatments, are generally considered to be aggressive competitors by growing very fast along with the developing root system of the treated plants and rapidly colonizing substrates to exclude pathogens (Papavizas, 1985; Howell, 2003). Furthermore, Green *et al.* (2001) explained the efficient biological control using *T. harzianum* by its ability to compete with *P. ultimum* for substrates from the seed coat and wounded or infected root tissues. Recently, Elshahawy and El-Mohamedy (2019) reported that in the greenhouse experiment, the combined inoculation of five *Trichoderma* isolates suppressed damping-off induced by *P. aphanidermatum* and increased the survival of tomato plants by 74.5%.

The mechanism of *T. harzianum* involved in the control of maize seedling disease caused by *P. ultimum*, investigated by proteome technique, revealed the capacity of *T. harzianum* strain T22 to not only promote seedling growth but also to induce the plant resistance through protein accumulation (Chen *et al.*, 2005). Also, biological control of damping-off of seeds and seedlings has been successfully accomplished to various degrees using the antagonistic fungus *G. virens* (Whipps and Lumsden, 1991).

In the current investigation, eight antagonistic bacteria were also tested for their ability to control the target pathogen. Results from the *in vitro* assay showed that even though no significant effect was noted against *P. ultimum* radial growth, microscopic observations made at the contact zone between the confronted agents revealed a strong alteration in pathogen hyphae mainly expressed as mycelium lysis and formation of mycelial cords. Contrarily, *Bacillus subtilis* and *Pseudomans fluorescens* isolated from the rhizospheric soil of healthy broccoli plants could completely inhibit *P ultimum* growth on PDA medium (El-Mohamedy, 2012). Besides, Idris *et al.* (2008) demonstrated that *B. cereus, B. subtilis, B. pumilus*, and *P. fluorescens* isolated from the rhizosphere of sorghum and grasses are able to suppress *P. ultimum in vitro* growth.

In the current study, all rhizobacterial agents tested as a seed treatment, excepting three isolates, had improved the emergence percentage of *P. ultimum*-inoculated and treated seedlings compared to pathogen-inoculated and untreated ones. However, when tested as root dipping, the reduction of the incidence of post-emergence damping-off ranged between 40 and 100% with *P. aureofaciens* 314 being the most efficient. Our results are in concordance with many previous studies showing that the use of *B. subtilis* and *P. fluorescens*, as soil mixing or root dipping treatments, might be used for controlling Pythium root rot on many crops (El-Mohamedy, 2012). Moreover, Khabbaz and Abbasi (2014) found through growth-room assays that *P. fluorescens* and *B. subtilis* are able to suppress Pythium damping-off and root rot of cucumber seedlings. Both pre- and post-planting application of these bacteria to an infested peat mix significantly increased percentage of healthy seedlings by 100-290%, and decreased damping-off and root rot severity by 27-50%. In addition, Naseby, Way*, et al.* (2001) showed that *Pseudomonas* strains decreased the number of lesions as well as root and soil *Pythium* populations. Other works also demonstrated the effectiveness of *P. putida*, *P. aureofaciens* and *P. aeruginosa* in suppressing Pythium damping-off in tomato (Buysens *et al.*, 1996). Parke (1990) also pointed

out that *Burkholderia cepacia* suppressed *P. ultimum* infections in pea in growth chamber experiments. Pythium root rot of wheat, attributed to *P. ultimum* and *P. irregulare*, is successfully controlled using *Bacillus* sp. strain L324-92 (Kim *et al.*, 1997). Idris *et al.* (2008) also showed the potential of *B. cereus, B. subtilis, B. pumilus* and *P. fluorescens* to efficiently control *P. ultimum* infection in sorghum probably due to their ability to produce antibiotic substances and siderophores as well as through the induction of systemic resistance. In addition, Georgakopoulos *et al.* (2002) found out that *Pseudomonas* spp. strains are the best candidates for controlling sugar beet damping-off incited by *P. ultimum* . In fact, *P. corrugata, P. fluorescens, P. marginalis, P. putida, P. resinovorans, P. syringae,* and *P. viridiflava* had significantly reduced the rate of decayed seeds due to *P. ultimum* infection. Among these microorganisms, *P. corrugata, P. fluorescens, P. marginalis, P. syringae*, and *P. viridiflava* had also significantly increased the percentage of emerged tomato seedlings (Gravel *et al.*, 2005). Besides, *B. subtilis* was ranked as an effective microorganism due to its ability to suppress root rot of cauliflower seedlings caused by *P. ultimum* var. *ultimum* (Abdelzaher, 2003).

In the present study, the rhizobacterial isolates *P. aureofaciens* 314, *P. aureofaciens* 31, *B. pumilus* 420, *P. fluorescens* and *P. putida* 227 showed growth promoting potential by increasing the seedling fresh weight by 73.06, 61.18, 77.39, 61.8 and 67.93%, respectively, over pathogen-inoculated and untreated control. Furthermore, *P. aureofaciens* 314 and *B. pumilus* 420 improved seedling height by 35.38 and 28.51%, respectively, as compared to control. These findings are in agreement with previous studies. *Pseudomonas* strains tested by Naseby, Way, *et al.* (2001) are also shown able to increase pea shoot and root weights as compared to *Pythium*-inoculated control. Furthermore, pre- and post-planting application of *P. fluorescens* and *B. subtilis* to an infested peat mix significantly had also increased cucumber plant fresh weights by 113-184% over control (Khabbaz and Abbasi, 2014).

These inhibitory and growth-promoting abilities may be achieved through various mechanisms of action. *P. fluorescens* can inhibit the germination of *Pythium* oospores, its growth, and subsequently the infection process (Ellis *et al.*, 1999). The mechanism by which *P. fluorescens* exerts its antagonism against *Pythium* sp. has been extensively studied and appears to involve the production of a variety of antibiotic compounds (Howell, 1980), competition (Ellis *et al.*, 1999) and induced host resistance (Benhamou *et al.*, 1996; Ramamoorthy *et al.*, 2002).

CONCLUSION

Pythium damping-off of pepper is a serious soilborne disease in Tunisia. As an eco-friendly alternative to chemical fungicide, the management of this disease through biocontrol agents is possible and results from the present study are promising. Some of the tested fungal and bacterial agents tested, were effective in suppressing *P. ultimum* mycelial growth and pepper damping-off disease and in improving plant growth.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

AUTHORS CONTRIBUTIONS

All the authors contributed equally to this work.